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TITLE: IDENTIFICATION OF WIDELY APPLICABLE TUMOR-ASSOCIATED ANTIGENS FOR BREAST CANCER IMMUNOTHERAPY

PRINCIPAL INVESTIGATOR: Jining Bai, Ph.D.

CONTRACTING ORGANIZATION: Johns Hopkins University
Baltimore, MD 21205

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Jining Bai, Ph.D.

E-Mail: jnbai@jhu.edu

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14. ABSTRACT
This study aims to identify widely applicable TAAs for immunotherapy against breast cancer. The rationale is to screen for candidate genes that are commonly over-expressed in human breast cancers, but not in benign breast tissue. These genes may reasonably serve as targets for antigen-specific immunotherapy against breast cancer. The study began with pp32 gene family members, which had been shown differentially and alternatively expressed in most human breast cancers. In general, benign breast tissues express pp32, a tumor suppressor, whereas breast cancers express tumorigenic family members, including pp32rl and pp32r2. The purpose of this study is to identify tumor-associated antigens (TAA) in pp32rl and pp32r2, then test their suitability as immunotherapeutic targets in breast cancer. In addition to pp32 gene family, BCOX1, BCOX2, and EBP50 were also identified as potential target for breast cancer immunotherapy.

TABLE OF CONTENTS

FINAL SCIENTIFIC REPORT

DAMD17-01-1-0280

Introduction:
In the IDEA proposal, we proposed a feasibility study of a novel immunotherapeutic strategy for the treatment of breast cancer. The rationale is based upon findings that genes belonging to the pp32 family are differentially and alternatively expressed in most human breast cancers. In general, benign breast tissues express pp32, a tumor suppressor, whereas breast cancers express tumorigenic gamily members, including ppewr1 and pp32 r2. Since pp32r1 and pp32r2 are expressed in nearly all breast cancers, but not in normal adult tissues, they may reasonably serve as targets for antigen-specific immunotherapy.

Body:

Statement of Works:

Task I. Identify, synthesize and test candidate peptides that could potentially bind to HLA class I molecules based on the coding sequence of pp32rl and pp32r2. (Month 1-6)

Task 2. Screen *in vitro* for candidate pp32rl & pp32r2 peptides that fulfill the requirements for TAA. (Month 7-12)

Task 3. Evaluate the pp32rl/pp32r2- specific cytotoxicity against a broad range of natural targets (established or primary breast cancer cell lines) to determine range of applicability.(Month 13 -20)

Task 4. Evaluate *in vivo* immunogenicity of pp32rl and/or pp32r2-derived TAAs in human breast cancer animal models. (Month 21-36)

1) Task #1: Identify, synthesize and test candidate peptides that could potentially bind to HLA class I molecules based on the coding sequence of pp32r1 and pp32r2.
Using Bioinformatics and ImmunoGenetics tools, we analyzed the entire coding region of pp32, pp32rl and pp32r2 genes for binding affinity with HLA-A*0201 molecule as well as the degradation pattern by proteasomal cleavages. The result of calculation shown (Table 1) that 19 motifs are potentially favorable of binding to HLA-A*0201 molecule with high affinity. To verify the prediction *in vitro,* HLA-A*0201+ TAP-deficient T2 hybridoma (ATCC) was pulsed with 50ug/ml of each peptide representing the motif (or control) and 5ug/ml ofb2-

microglobulin for 18hr at 37 C. HLA-A*0201 expression was then measured by flow cytometry using mAb BB7.2 (ATCC) followed by incubation with FITC-conjugated secondary antibody. Fluorescent index of HLA-A*0201 to each peptide can be determined as: (mean fluorescence with peptide - mean fluorescence without peptide) / (mean fluorescence without peptide). The result shown 10
out of 20 motifs is capable of binding to HLA-A*0201 in a concentration dependent manner (Table 1).

2) Task #2: Screen for candidate pp32r1 & pp32r2 peptides that fulfill the requirements for TAA.

In order to be qualified as a TAA, a motif has to be able to meet several criteria in addition to the binding to HLA-A*0201. These requirements include (i) the antigen can be naturally processed by tumor cells, (ii) it permits expansion of antigen-specific CTL; (iii) it is presented in a MHC restricted fashion. CTL assay was carried out to test if the motifs identified in Aim#1 fulfill the requirements for TAA. In brief, Cr^{51}"-labeled target cells (T2 cells pulsed with peptide or cancer cell expressing pp32 family members) were incubated with various numbers of CTL effecter cells for 4 hr. Cr^{51}- release assays were performed in triplicate per condition using 5x10^ labeled target cells per well in a 96-well plate. Percent specific lysis will be calculated from CPM of (experimental result
- spontaneous release)/(maximum release - spontaneous release). The results, summarized in Table 2, indicate that 2 out of 10 motifs fulfilled the above requirement as TAA.

3) Task #3. Evaluate the applicability of the pp32r1/pp32r2- specific cytotoxicity against a broad range of natural targets.

To evaluate the applicability of the pp32r1/pp32r2- specific cytotoxicity against a broad range of natural targets, primary cultures of breast tumor that are both HLA-A*0201 positive and pp32r1 /pp32r2 positive was selected as target cells. The expression of HLA-A*0201 was verified by flow cytometry, whereas the expression of pp32r1 and/or pp32r2 was confirmed by subtype specific RT-PCR. CTL assay was carried out to test if the motifs identified in Task #1 &#2 are applicable to HLA-A*0201 positive and pp32r1 / pp32r2 positive primary cultures. In brief, Cr^{51}-labeled target cells were incubated with various numbers of CTL effecter cells for 4 hr. Cr^{51}-release assays were performed In triplicate per condition using 5x10^ labeled target cells per well in a 96-well plate. Percent specific lysis will be calculated from CPM of (experimental result- spontaneous release)/(maximum release - spontaneous release). Unlike the artificial target cells used in Aim#2, the results shown no detectible pp32r1/pp32r2- specific cytotoxicity against primary cultures of breast tumor that are both HLA-A*0201 positive and pp32r1 / pp32r2 positive. A possible explanation might be the difference in expression/presentation of pp32r1 /pp32r2 between primary cells and artificial target cells. Due to the high homology among pp32 family members (over 90% identity at amino acid level), none of the existing antibodies is subtype- specific. Therefore, the reliable method to screen pp32r1 and pp32r2 expression has been based on RT-PCR. Although this screen method is

very effective to identify cells/tissue that express pp32r1 and pp32r2 at mRNA level, its result may not correlate with the expression of pp32r1 and pp32r2 at the protein level, which is crucial for evaluating pp32r1/pp32r2- specific cytotoxicity.

4) Task#4. Evaluate *in vivo* immunogenicity of pp32r1 and/or pp32r2-derived TAAs in human breast cancer animal models.
Due to the challenges described in Task#3, we modified the target tumor cells used in our animal models. We made GFP-pp32r1 and GFP-pp32r2 fusion constructs in mammalian expression vector pEFPN1. Stable transfectants with GFP-pp32r1 and GFP-pp32r2 expression were isolated by FACS selection. This modification enabled us to circumvent the challenges described in Task #3 and allow us to trace the target tumor cells and its metastasis in vivo. However, our study based on the modified design shown no detectable anti-tumor activity of pp32r1/pp32r2- specific CTLs in breast cancer xenograft model. This outcome is likely due to the caveat that the antigens from GFP-fusion proteins might be processed or presented differently than those from unmodified pp32r1 and pp32r2 in the target cancer cells.

5) Alternative Task:
During the No Cost Extension period, we carried out studies to identify other candidate genes that may serve as potential target in breast cancer immunotherapy described as backup approach in the proposal).
Based on SAGE analysis, we were able to generate a differential expression-based classification map on breast tumor vs benign tissue.
The correspondent gene to the reliable UniGene clusters matched to the identified sage tag was cloned. And the differential expression profile of the cloned gene was further examined by in situ hybridization and immunohistochemistry in breast cancer specimen. With this strategy, we were able to identify three candidate genes (BCOX1, BCOX2 and EBP50) that may serve as potential targets in breast cancer immunotherapy (Song, et al 2006, 2007)

Key Research Accomplishments:

We have identified two peptide motifs from pp32 family members, which fulfill the requirement to be TAAs. This study provided bases for further feasibility study of pp32r1 and pp32r2 as target breast cancer immunotherapy.

Reportable Outcomes:

The result of Specific Aim #1 and #2 were presented at 2002 Era of Hope Department of Defense Breast Cancer Research Program Meeting.

Yu, W., Jagun, A., Zhu, X., Jaffee, EM, & Bai, J. Identification of Candidate Tumor-Associated Antigens from pp32 Family Members. *Era of Hope* (BCRP): 3:54-2, 2002.

Song, J, Yang, W, Shih, IeM, Zhang, Z, Bai, J: Identification of BCOX1, a novel gene overexpressed in breast cancer. Biochim Biophys Acta. 2006 Jan;1760(1):62-9. **[Appendix A]**

Song, J, Bai, J, Yang, W, Gabrielson, EW, Chan, DW, and Zhang, Z: Expression and clinicopathological significance of oestrogen-responsive ezrin–radixin–moesin-binding phosphoprotein 50 in breast cancer. Histopathology 2007; 51 (1): 40-53. **[Appendix B]**

Conclusions:

We demonstrated *in vitro* that:

(i) the oncogenic pp32 family members can be presented by HLA-A*0201,
(ii) the HLA-A*0201 cells bearing these motifs can be recognized and lyzed by pp32r1-or pp32r2-specific CTL in a MHC class I specific manner.
(iii) Other candidate genes (BCOX1, BCOX2 and EBP50) may serve as potential targets in breast cancer immunotherapy.

Summary of Personnel Partially Supported by this Idea Award:

1. Jining Bai (PI)
2. Elizabeth Jaffee (Co-Investigator)
3. Xianzheng Zhou (Co-Investigator)
4. Jin Song (Postdoctoral Fellow)
5. Tianzhi Mao (Technician)
6. Wanjun Yang (Technician)
7. Ghasson Ibrahim (Technician)
8. Adetinuke Jagun (Technician)
9. John Lee (Technician)

Appendix A

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Identification of BCOX1, a novel gene overexpressed in breast cancer

Jin Song [a], Wanjun Yang [a], Ie-Ming Shih [a,b], Zhen Zhang [a], Jining Bai [a,*]

[a] *Department of Pathology, Johns Hopkins Medical Institutions, 417 N. Caroline Str., Baltimore, MD 21231, USA*
[b] *The Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins Medical Institutions, Baltimore, MD 21231, USA*

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Abstract

The identification of tumor-associated antigens, which are specifically expressed in cancer tissues, is of utmost important for immunotherapy of breast cancer. We have combined in silico screening and experimental expression analysis to identify genes that are differentially expressed in breast carcinomas compared with their corresponding normal tissues. Using these approaches, we identified a novel gene, BCOX1, with overexpression in breast carcinoma. BCOX1 was highly homologous to KIAA0100, a hypothetical gene located on chromosome 17q11.2. RNA in situ hybridization shows that BCOX1 mRNA signal is mainly located in the cytoplasm of breast carcinoma epithelial cells, but not in those of normal epithelial cells, stroma cells and lymphocytes. Furthermore, mRNA expression of BCOX1 was moderately elevated in ductal in situ carcinoma (DCIS), peaked in invasive breast carcinoma (IBC) and metastatic breast carcinoma cells (MET) whereas absent in benign ductal epithelial cells. The predicted BCOX1 open reading frame of 666 bp encodes a putative protein of 222 amino acid residues with a calculated molecular weight of 24920 Da and a PI of 5.86. Computational analyses predict that the putative BCOX1 protein is a cytoplasmic protein. The functional relevance of this novel gene is yet to be determined. This study warrants further investigations to explore the molecular functions of BCOX1, and to determine its potential diagnostic and therapeutic applications for breast cancer.

Keywords: SAGE; EST database; Gene characterization; Cancer

1. Introduction

Breast carcinoma is the second leading cause of cancer-related death in women in the United States [1]. Despite improvements in cancer therapy, about one quarter of the patients diagnosed with invasive breast carcinomas will eventually die from their diseases. Thus, the identification of novel breast cancer markers and tumor-associated antigens (TAA) are of utmost important for improvement in early diagnosis of breast carcinomas and for promising immunotherapy strategy [2].

Recently, the sequence data in the public databases and the rapid advancement in the field of bioinformatics have provided researchers excellent approaches to systematically analyze and identify genes that could be used as tumor diagnostic markers, prognostic indicators, and suitable targets for various forms of therapeutic intervention [3–5]. Serial analysis of gene expression (SAGE) and expressed sequence tag (EST) are the two major sources of expression data in the public domain. Analysis of these cDNA data has been proved to be an effective method of identifying and characterizing genes expressed in a variety of human tissues and in different pathological situations [3–5].

To identify the genes, which are differentially expressed in breast carcinomas compared with their corresponding normal tissues, we screened SAGE and EST databases, and narrowed down our research interest on a set of specific SAGE tags for breast carcinomas. To validate the correlation of expression profiles between the SAGE tags and the corresponding genes or hypothetical genes, RT-PCR was carried out using cDNA generated from matched normal/tumor tissues panel. Furthermore, RNA in situ hybridization was used to examine the tissue expression patterns of the target gene on a panel of benign and tumor breast tissues. In this study, we report the identification and characterization of a novel tumor-associated gene, BCOX1 [GenBank: AY943906], in breast carcinoma.

Abbreviations: BCOX1, breast cancer overexpressed gene 1; SAGE, serial analysis of gene expression; EST, expressed sequence tag; RT-PCR, reverse transcription-polymerase chain reaction

* Corresponding author. Tel.: +1 410 955 6920; fax: +1 410 502 5158.
E-mail address: jnbai@jhmi.edu (J. Bai).

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2. Materials and methods

2.1. Specimens and chemicals

Archival specimens as formalin-fixed, paraffin-embedded blocks were retrieved from the Surgical Pathology at Johns Hopkins Medical Institutions (JHMI), and prepared at the reference lab at JHMI. The acquisition of human tissue material was approved by the local Institutional Review Board. The panel of specimens included 14 benign breast tissue specimens (BBT), 12 ductal carcinomas in situ (DCIS), 17 invasive breast carcinomas (IBC), and 14 metastatic breast carcinomas (MET), which were obtained during the period of 1998 to 2003 from patients with or without diagnoses of breast carcinomas undergoing reduction mammoplasty and mastectomy. For each specimen selected, sections were subjected to H&E staining and reviewed by a surgical pathologist (I-M.S.) to confirm the diagnosis. All cases were characterized for clinical–pathologic parameters, including histological type, grading, tumor size and nodal involvement. Partial cases were assessed for biological parameters, including steroid hormone receptors (estrogen receptor, ER and progesterone receptor, PR), HER2/neu status and cell proliferation (Ki-67 index). The breast carcinomas were classified according to the 1999 WHO criteria.

Restriction enzymes (*Eco*RI, *Xho*I, and *Bam*HI), and Taq DNA polymerase were purchased either from Invitrogen Corporation (Carlsbad, CA) or from New England BioLabs Inc. (Beverly, MA). Dig RNA labeling kit (SP6/T7), Anti-digoxigenin-AP Fab fragments, and Nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) stock solutions were purchased from Roche Diagnostics Corporation (Indianapolis, IN). All other chemicals of molecular biology grade were obtained from Sigma-Aldrich (St. Louis, MO).

2.2. Bioinformatics

UniGene clustering, chromosomal maps and mRNAs aligning to the Human Genomic Contig were investigated with biocomputing services of the National Center for Biotechnology Information (NCBI), such as SAGEmap, Evidence Viewer, MapView, and Model Maker et al. (http://www.ncbi.nlm.nih.gov/projects/SAGE/). The putative coding sequences were predicated using Open Reading Frame (ORF) Finder of NCBI. To obtain possible orthologous sequences of BCOX1 from different species, various sequence databases were searched using TBLASTN program at NCBI with the human protein sequence as a query. Derived protein sequences were analyzed using various bioinformatics tools: SignalP for signal peptide prediction [6], PSORT and TargetP for cellular localization prediction [7,8], ScanProsite for potential specific motif scanning [9], and ClustalX for multiple sequence alignment (http://us.expasy.org/) [10].

2.3. Reverse transcription-polymerase chain reaction (RT-PCR)

The template was cDNA from 16 different human tissues (Human Multiple Tissue cDNA/MTC Panels, BD Biosciences, Clontech, Palo Alto, CA) and from 4 pairs of human breast carcinoma and corresponding normal tissue of individual patient (Human Breast Matched cDNA Pair Panel, Clontech, Palo Alto, CA), respectively. The PCR amplifications were carried out in a ThermoHybaid PCR Express (San Diego, CA) as follows: initial denaturation at 94 °C for 5 min; for MTC panels, 40 cycles of 40 s at 94 °C, 1 min at 62 °C, and 1:30 min at 72 °C; for Breast Matched cDNA Pair Panel, 35 cycles of 40 s at 94 °C, 1 min at 60 °C, and 1:30 min at 72 °C; followed by a final 5 min extension at 72 °C. Amplified PCR products were electrophoresed on a 1% agarose gel and visualized by ethidium bromide staining.

The primer design for general PCR is described as the following: For MTC panels, BCOX1 PCR product of 375 bp, upper primer: 5′-CGT GGT ATT GTC CAT GAA TAG T-3′, lower primer: 5′-GAA GGG CAA GAG GTA AAA CTG-3′. For breast matched cDNA pair panel, BCOX1 PCR product of 452 bp, upper primer: 5′-GGT GGA TCA AAA GGA ACT GTC-3′, lower primer: 5′-TTG GCT CAA CTA AGT TTT CTG T-3′; β-actin PCR product of 252 bp, upper primer: 5′-AGG CAT CCT CAC CCT GAA GT-3′, lower primer: 5′-CCA GAG GCG TAC AGG GAT A-3′. All primers were purchased from Qiagen (Valencia, CA).

2.4. Full-length cDNA cloning of BCOX1

To isolate the entire coding region cDNA fragment of BCOX1, we employed a nested PCR method to amplify cDNA fragments of BCOX1 from the breast cancer tissue cDNA pool. The primer sequences for nested PCRs were: For the first PCR, PCR product of 1106 bp, upper primer: 5′-GGG AGG TTG TTG CTG AAG T-3′, lower primer: 5′-GGG GCT ATT TTC TCT CAG TAT G-3′; For the second PCR, PCR product of 685 bp, upper primer: 5′-*C GAATTC AT* ATG GTT CTC AAG GTG GAT ACC TCT-3′, lower primer: 5′-*C GAATTC* TTA AGG AGA CCA TAT GGG GCA T-3′. The PCR conditions were: For the first PCR, initial denaturation at 94 °C for 5 min; 50 cycles of 40 s at 94 °C, 1 min at gradient 55–65 °C, and 1:30 min at 72 °C; followed by a final 5 min extension at 72 °C. For the second PCR, initial denaturation at 94 °C for 5 min; 2 cycles of 40 s at 94 °C, 1 min at gradient 58–68 °C, and 1:30 min at 72 °C; followed by 40 cycles of 40 s at 94 °C, 1 min at 68 °C, and 1:30 min at 72 °C; followed by a final 5 min extension at 72 °C. The amplified full-length cDNA of BCOX1 was subcloned into the IPTG-inducible bacterial expression vector pGEX2T (Amersham-Biosciences, Piscataway, NJ) according to the manufacturer's protocol. Automated sequencing of the PCR product and representative inserts was performed by the DNA Core Facility at JHMI.

2.5. Preparation of sense and antisense BCOX1 probes

The BCOX1 PCR product of 452 bp from above RT-PCR for Breast Matched cDNA Pair Panel was purified using QIAquick PCR Purification Kit (Qiagen, Valencia, CA). This purified cDNA fragment was subcloned into the transcription vector pCR®II-TOPO® (Invitrogen, Carlsbad, CA) and transformation of TOP10 cells was performed according to the manufacturer's protocol. Several clones were analyzed for the presence of a BCOX1 insert by digestion with *Eco*RI on the purified plasmid DNA (QIAprep Spin Miniprep kit, Qiagen). Automated sequencing of the representative inserts was performed by the DNA Core Facility at JHMI.

To generate the antisense (complementary to BCOX1 mRNA) and sense RNA probes, the purified plasmid DNA was firstly linearized with *Xho*I or *Bam*HI, respectively. Antisense and sense RNA probes labeled with digoxigenin-UTP were generated by in vitro transcription of purified linearized plasmids (QIAquick PCR Purification Kit, Qiagen) with SP6 or T7 RNA polymerase (Roche Diagnostics Corporation, Indianapolis, IN) according to manufacturer's protocol.

2.6. In situ hybridization

Formalin-fixed, paraffin-embedded tissue blocks were cut into 4-μm thick sections and were mounted on ProbeOn Plus slides (Fisher Scientific, Pittsburgh, PA). To control the signal specificity, two consecutive sections were used for hybridization with the antisense and sense probes, respectively. In situ hybridization was carried out with a nonradioactive method. Briefly, the sections were deparaffinized by submerging the slides in xylene; rehydrated in decreasing concentrations of ethanol (100% two times followed by one time each of 95% and 70%); and equilibrated, first in nuclease-free water and then in TBS buffer (pH 7.5). The tissue sections were subjected to protease K digestion (10 ng/μl) at 37 °C for 30 min in a humidified chamber. The slides were washed twice, 1 min each, with TBS buffer (pH 7.5), and incubated with 100 μl pre-hybridization solution without probe at 45 °C for 1 h in a humidified chamber. The pre-hybridization solution contains 50% formamide, 4× sodium saline citrate (SSC), 2× Denhardt's solution, 5× dextran sulfate, 0.1% sodium dodecyl sulfate (SDS), and 400 μg/ml denatured salmon sperm DNA. The 50-μl hybridization solution containing 1 ng/μl probe (sense or antisense) was applied to each section, and denatured at 65 °C for 5 min on the heat block. Hybridization was carried out at 54 °C overnight in a humidified chamber. The slides were washed with 2× SSC twice for 20 min, twice for 15 min, followed by one time wash in 2× SSC of 50 °C for 30 min.

2.7. Detection of digoxigenin-labeled probes

Detection of in situ hybridization probes was performed using anti-digoxigenin-alkaline phosphatase system (Roche Diagnostics Corporation,

Indianapolis, IN). Briefly, the sections were first equilibrated in TBS-T buffer for 5 min, and incubated with 1× blocking buffer (Roche) at room temperature for 30 min in a humidified chamber. The slides were then incubated with an antibody solution containing 1:500 diluted alkaline phosphatase conjugated Fab fragment from a sheep anti-digoxigenin antibody (Roche) at room temperature for 1 h 30 min. The sections were washed three times with TBS-T buffer for 10 min each on the shaker (100 rpm), and equilibrated in TBS buffer (pH 9.5) at room temperature for 5 min to activate alkaline phosphatase. Colorimetric detection of the digoxigenin labeled probe was performed by applying 100 μl of substrate solution containing NBT/BCIP per slide and incubated at room temperature for 3 h in a humidified chamber. Color development was terminated by two washes of 3 min each in nuclease-free water. The sections were dehydrated, and mounted with the supermount medium (Fisher Scientific, Fairlawn, NJ). A positive enzyme reaction in this assay stained dark purple. Staining intensity was graded from 0 to 3+ as follows: no staining (0), weak staining (1+), moderate staining (2+), and intense staining (3+).

2.8. Statistical analyses

The PCR band densities were measured using Quantity One-version 4.5 (Bio-Rad, Hercules, CA) and Student's *t* test was used to analyze the BCOX1 differential expression levels of between the paired breast carcinomas and their corresponding normal partners. Fisher exact test was performed to compare the BCOX1 differential expression levels in different categories of a panel of benign and tumor breast tissues.

3. Results

3.1. Computational analysis of the Unigene cluster Hs. 151761

Previously, we identified seventy-one cancer type-specific tags from the SAGE database, some of which had been selected to generate a gene expression-based classification map. This expression map had been demonstrated to provide a reliable and practical approach to determine tumor type in cases of metastatic carcinoma of clinically unknown origin [11]. Among 71 cancer type-specific tags, there are 28 specific tags for breast carcinomas. To identifying genes that were differentially expressed in breast carcinomas, all of the 28 tags were matched to the UniGene database. In this study, we focused on one tag of GGTCCCCTAC, which had been demonstrated highly expressed in the SAGE libraries of breast carcinomas and breast cancer cell lines compared to the SAGE libraries of normal mammary epithelium (Fig. 1, the gene expression profile chart generated from the NCBI Gene Expression Omnibus database). The reliable UniGene cluster matched to the tag of GGTCCCCTAC is Hs.151761 corresponding to a hypothetical gene, KIAA0100, which includes a total of 103 sequences mostly from EST database. KIAA-related EST clones were assigned to chromosome 17q11.2 {Contig: [GenBank: NT_010799]; Locus ID: 9703; RefSeq: [GenBank: NM_014680]}. The NCBI database search indicated that there are about 38 putative exons belonging to this hypothetical gene (KIAA0100) spanning 31,545 bp. The computational model of KIAA0100 generated from selected GenBank/ESTs sequences aligned to this region was shown in Fig. 2. To date, all of KIAA-related sequences were derived from EST sequences with few further reports in the literature [12].

3.2. Expression of BCOX1 in breast carcinomas and corresponding normal tissues

Due to the nature of KIAA0100 as a hypothetical gene as well as its apparent complex EST profile, it is necessary to examine the correlation of expression profiles of SAGE tag GGTCCCCTAC and KIAA0100-related sequences in breast carcinomas, as well as to characterize the KIAA0100 variant(s) that is responsible for the over-expression in breast carcinomas. Gradient PCR were first performed to optimize the annealing temperature for different primer sets using human placenta cDNA as template. And these primer sets were then used to amplify the specific fragments derived from different variants of KIAA0100, which includes {[GenBank: NM_014680], [GenBank: D43947], [GenBank: AK130007], [GenBank: BC050440] and [GenBank: BC048096]}, using human breast matched cDNA pair panel as template. A single PCR product {one fragment derived from [GenBank: BC048096], an EST clone isolated from hippocampus} was detected in breast cancer tissues, whereas the other KIAA0100 variants related fragments were not detectable (data not shown). We subsequently cloned the cDNA with complete coding region from human breast cancer tissue and deposited the sequence data in GenBank database [GenBank: AY943906].

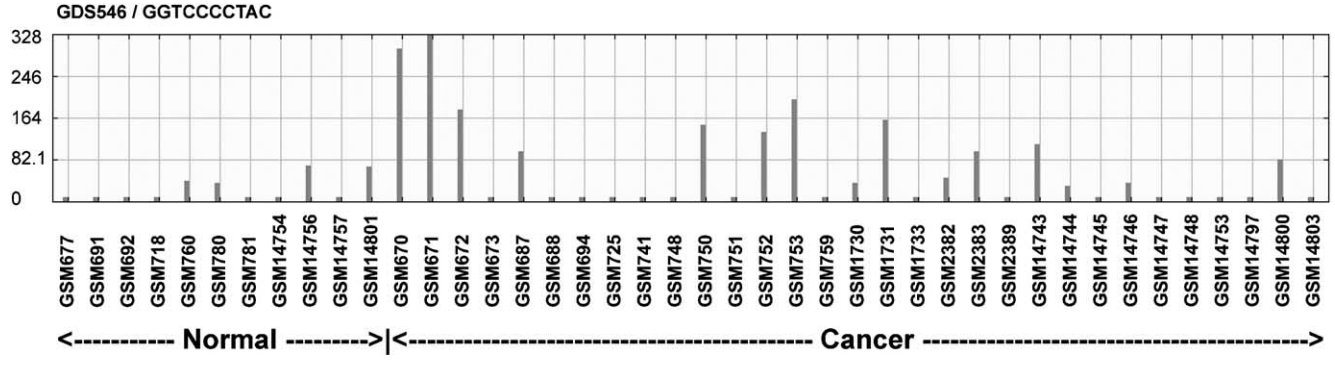

Fig. 1. Expression profile chart of the SAGE Tag of GGTCCCCTAC. The gene expression profile chart was generated from the NCBI Gene Expression Omnibus database (GEO). There are a total of 42 samples, including 11 normal breast tissues and 31 breast carcinomas and breast cancer cell lines, within the GDS546 of Cancer Genome Anatomy Project (CGAP) SAGE library collection. The *x* axis represents the GEO accession number, which are corresponding to different SAGE libraries. The *y* axis represents the log-transformed values of user-provided normalized signal count data for single-channel data, which reflects the relative measure of the abundance of each transcript.

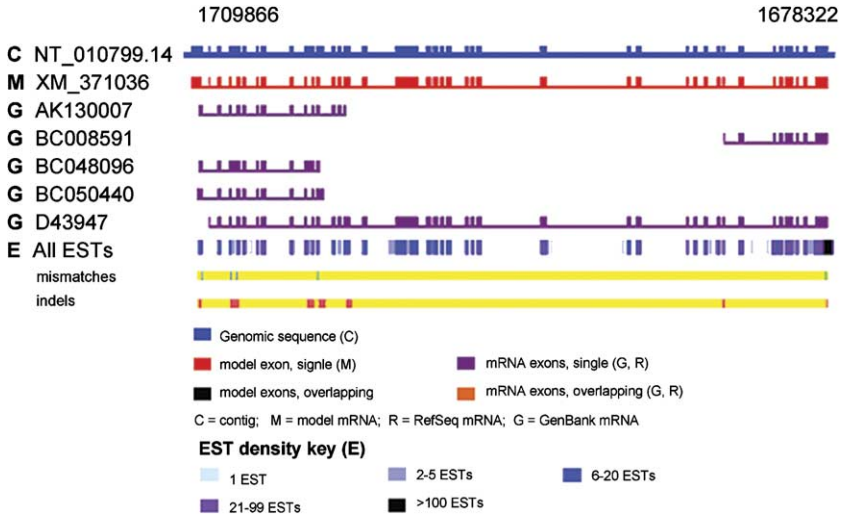

Fig. 2. The computational model of KIAA0100 and its related sequences. The display was produced using the Evidence Viewer of NCBI. The numbers just above the blue bar are the coordinates of the 5′ and 3′ extremes of the gene on the contig. There are about 38 exons belonging to one hypothetical gene (KIAA0100) in this genomic region spanning 31,545 bp. The computational model generated from selected GenBank entry of IMAGE clone and all available ESTs aligning in this region was shown. The mismatches and indel lines indicate that the positions of any mismatches, insertion/deletions (indels), or NM/mRNA sequence gaps in any of the NM/mRNA alignments for a given exon.

To quickly determine if this gene/variant is expressed in breast carcinomas and their corresponding normal tissues, we performed RT-PCR on a panel of cDNA isolated from 4 matched tissue pairs of human breast carcinoma and corresponding normal tissue. As shown in Fig. 3A and B, one band with expected size of 452 bp is detected in all of lanes (lanes C1 to N1), and its expression level in breast carcinomas (lanes C1 to C3) is much higher than that of corresponding normal breast tissues (lanes N1 to N3) respectively. The expression level of β-actin was taken as a control for this comparison, which is consistent across the tissue pairs. Statistical analysis based on the relative ratios of BCOX1/β-actin (band densities) shows there is a significant difference of this gene expression levels of between the paired breast carcinomas and their corresponding normal partners (P= 0.03). Because of its overexpression in breast carcinomas compared with their corresponding normal tissues (considering together with the following results of RNA in situ hybridization), this gene was named BCOX1. To avoid the limitation inherited by PCR, we decided to carry out further study at next level using in situ hybridization analysis.

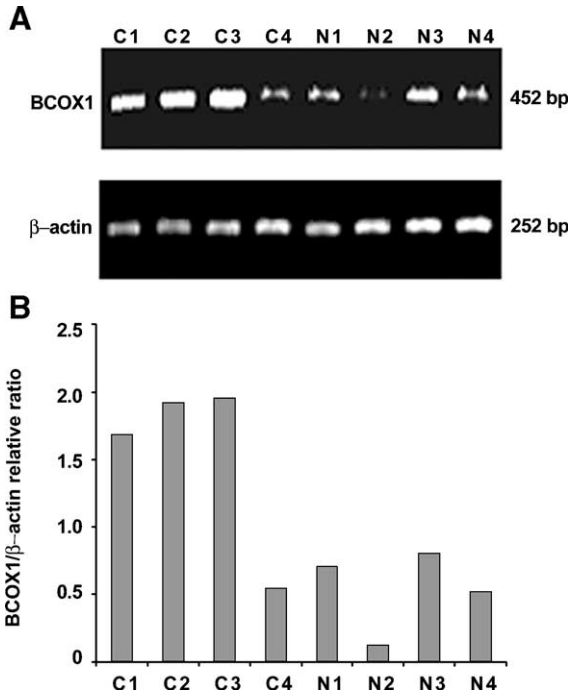

Fig. 3. BCOX1 expression in breast carcinomas and corresponding normal tissues. (A). PCR was carried out on the panel of cDNA isolated from 4 pairs of human breast carcinoma and corresponding normal tissue of individual patient (Human Breast Matched cDNA Pair Panel). One specific band with expected size of 452 bp (corresponding to BCOX1, but not the predicated KIAA0100 transcript) was detected in all of the lanes (lanes C1 to N4), and its expression level is elevated in breast carcinomas (lanes C1 to C3) compared with corresponding normal breast tissues (lanes N1 to N3) respectively. However, the expression level of β-actin taken as a control, one band about 252 bp in size, is very consistent across the tissue pairs. (B) The relative ratios of BCOX1/β-actin expression levels (band densities) were shown in the histogram. Statistical analysis shows there is a significant difference of BCOX1 expression levels of between the paired breast carcinomas and their corresponding normal partners (P=0.03).

3.3. In situ hybridization analysis

To further investigate the tissue expression pattern of BCOX1 gene in breast tissues, we performed RNA in situ hybridization analyses on a panel of specimens included BBT, DCIS, IBC, and MET. BCOX1 mRNA expression was visualized by in situ hybridization using a digoxigenin labeled BCOX1 antisense probe. BCOX1 sense probe was used as a negative control for every specimen by hybridization on a consecutive section. For each category, one set of representative staining was shown in Fig. 4. The experimental results showed apparently cytoplasmic staining in breast carcinoma epithelial cells, but not in normal ductal epithelial cells, stroma

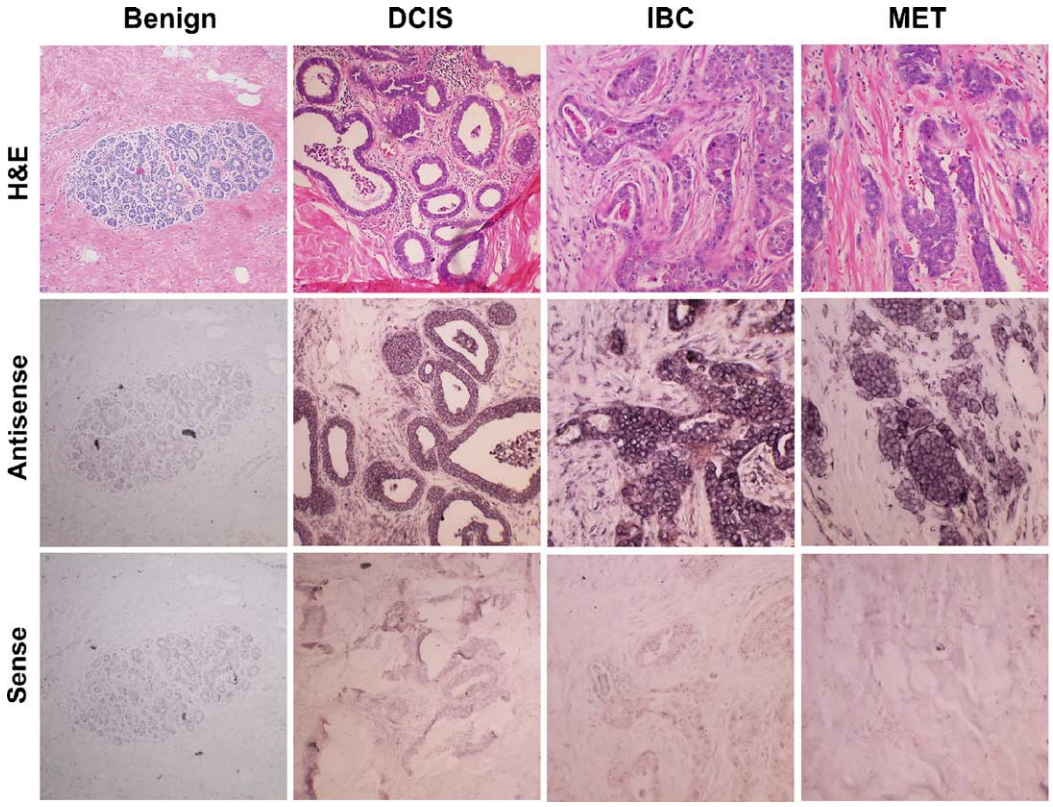

Fig. 4. RNA in situ hybridization analysis of BCOX1 in breast carcinomas. BCOX1 mRNA expression was visualized by in situ hybridization using a digoxigenin labeled BCOX1 antisense probe. A BCOX1 sense probe was used as a negative control for every specimen by hybridization on a consecutive section. For each group, one set of representative staining, including H&E, antisense and sense were shown in Fig. 4. Top panel, H&E-stained tissue sections; middle panel, moderate and strong cytoplasmic staining with BCOX1 antisense probe for DCIS, IBC and MET group, but absent staining for benign group; bottom panel, no signal in control staining with BCOX1 sense probe for all. All, ×200 magnification.

cells and lymphocytes. Furthermore, moderately elevated signal was observed in ductal in situ carcinoma and peaked in invasive and metastatic breast cancer cells, (the middle panels of DCIS, IBC and MET in Fig. 4), whereas absent staining in benign breast tissues (the middle panel of benign in Fig. 4). No specific staining was observed in controls that were hybridized with BCOX1 sense probe (the bottom panel of benign, DCIS, IBC and MET in Fig. 4). Table 1 further demonstrates that mRNA expression of BCOX1 were significantly higher in tumor breast tissues compared to benign breast tissues ($P=0.004$ or $P\leq 0.0001$), and mRNA expression of BCOX1 were also significantly higher in IBC ($P=0.0019$) and MET ($P=0.004$) compared to DCIS. Breast carcinomas with lymph node involving show significantly stronger staining of BCOX1 than those without lymph node involving ($P=0.0081$), whereas there were no significantly correlations of between mRNA expression of BCOX1 and other clinical–pathologic parameters (Table 2). These results indicated that the BCOX1 gene was differentially expressed in breast carcinomas, and could be used potentially as a biomarker for diagnosis or a target for therapeutic intervention of breast cancer.

Table 1
BCOX1 mRNA expression pattern in benign and tumor breast tissues

Tissues	*n*	Absent	Weak/moderate	Intense	*P* value
Benign Breast	14	14 (100%)	0 (0%)	0 (0%)	
DCIS	12	6 (50%)	6 (50%)	0 (0%)	0.004
IBC	17	0 (0%)	9 (52.9%)	8 (47.1%)	≤0.0001
MET	14	0 (0%)	4 (28.6%)	10 (71.4%)	≤0.0001

n represents total sample number for each category. '*xx* (*xx*%)' represents 'the number of positive staining cases (positive cases percentage)'. *P*'s were calculated by Fisher exact test. Level of significance in each category was determined by comparing with benign breast samples.

3.4. Restricted expression of BCOX1 in normal tissues

To determine experimentally the relative expression profile of BCOX1 mRNA in different normal tissues, we performed RT-PCR on the panels of cDNA isolated from a number of normal tissues including the heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, colon, and peripheral blood leukocyte. As shown in Fig. 5, a specific band about 375 bp in size is detected in placenta and pancreas, indicating that this gene is also selective expressed in these tissues.

3.5. Full-length cDNA of BCOX1 and putative amino acid sequence

The complete nucleotide sequence of full-length cDNA of BCOX1 cloned from human breast cancer tissue was con-

Table 2
Correlation between BCOX1 mRNA expression and clinico-pathologic variables in breast carcinomas

Variables	*n*	Absent	Weak/moderate	Intense
Histotype				
Ductal	39	6 (15.4%)	17 (43.6%)	16 (41%)
Lobular	4	0 (0%)	2 (50%)	2 (50%)
Elston grade				
I	1	0 (0%)	1 (100%)	0 (0%)
II	20	2 (10%)	10 (50%)	8 (40%)
III	22	4 (18.2)	6 (27.3%))	12 (54.5%)
Lymph node status				
N−	29	6 (20.7%)	15 (51.7%)	8 (27.6%)
N+	14	0 (0%)	4 (28.6%)	10 (71.4%)*
Steroid receptor				
ER−	8	0 (0%)	1 (12.5%)	7 (87.5%)
ER+	20	0 (0%)	10 (50%)	10 (50%)
PR−	14	0 (0%)	4 (28.6%)	10 (71.4%)
PR+	15	0 (0%)	8 (53.3%)	7 (46.7%)
Cell proliferation				
Low Ki-67	20	0 (0%)	10 (50%)	10 (50%)
High Ki-67	8	0 (0%)	2 (25%)	6 (75%)
HER2/neu status				
HER2/neu−	18	0 (0%)	7 (38.9%)	11 (61.1%)
HER2/neu+	6	0 (0%)	4 (66.7%)	2 (33.3%)

n represents total sample number for each category.
'*xx* (*xx*%)' represents 'the number of positive staining cases (positive cases percentage)'.
* *P* value was calculated by Fisher exact test, $P=0.0081$. Level of significance in each category was determined by comparing with its respective partner.

```
378 atggttctcaaggtggatacctctgagtccttatggcatattcag
    M  V  L  K  V  D  T  S  E  S  L  W  H  I  Q
423 atcagtagaagcagatttcttttggatagtgatgggaaaaggcta
    I  S  R  S  R  F  L  L  D  S  D  G  K  R  L
468 atctgtgaggtgagcttatgtaagatcaacagcaaagttctaaag
    I  C  E  V  S  L  C  K  I  N  S  K  V  L  K
513 agtggtcagctggaggacacctgcctagtggagctttcactggcc
    S  G  Q  L  E  D  T  C  L  V  E  L  S  L  A
558 ctggacctgtgtctaaaggtgggcattagcagtcggcatctcact
    L  D  L  C  L  K  V  G  I  S  S  R  H  L  T
603 gctatcactgtggatgtgtggacactccatgctgaactgcatgag
    A  I  T  V  D  V  W  T  L  H  A  E  L  H  E
648 ggcctcttccagagccaactgctgtgccagggcccaagcctagca
    G  L  F  Q  S  Q  L  L  C  Q  G  P  S  L  A
693 tctaagcctgttccctgttcagaggtgacagaaaacttagttgag
    S  K  P  V  P  C  S  E  V  T  E  N  L  V  E
738 ccaactctgcctggcctattccttctccagcagctgccagaccag
    P  T  L  P  G  L  F  L  L  Q  Q  L  P  D  Q
783 gtcaaggttaagatggagaacacaagcgtggtattgtccatgaat
    V  K  V  K  M  E  N  T  S  V  V  L  S  M  N
828 agtcaaaagaggcacctgacttggactctgaagctgctgcagttc
    S  Q  K  R  H  L  T  W  T  L  K  L  L  Q  F
873 ctgtaccaccgtgatgaggatcagctgccccttcgaagcttcaca
    L  Y  H  R  D  E  D  Q  L  P  L  R  S  F  T
918 gcaaactctgatatggcacagatgagcactgaactgctgctggaa
    A  N  S  D  M  A  Q  M  S  T  E  L  L  L  E
963 ggttcatgggtcggggtactggtactgagcgagaatgggaggact
    G  S  W  V  G  V  L  V  L  S  E  N  G  R  T
1008 agatactctctttgctcatgccccatatggtctcctaa 1046
     R  Y  S  L  C  S  C  P  I  W  S  P  *
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Fig. 6. The complete coding sequence of BCOX1 and putative amino acid sequence. The open reading frame of BCOX1 and putative amino acid sequence were predicted using ORF Finder of NCBI. The complete coding sequence (CDS) is about 666 nucleotides which codes a putative protein consists of 222 amino acid residues with a molecular weight of 24920 Da and a PI of 5.86. The translational start codon and stop codon are indicated in bold. The carboxyl-terminus of about 32 amino acid residues indicated in italicized letters is unique for BCOX1.

firmed by bi-directional sequencing the PCR product and representative inserts, and revealed that it was a new gene differentially expressed in breast carcinomas [GenBank: AY943906]. BCOX1 sequence is 1103 bp in length, containing 666 nucleotides in the putative coding region, flanked by 377 bp in the 5′-untranslated region and 60 bp in the 3′-untranslated regions. Compared to other variants of the hypothetical KIAA0100 gene, BCOX1 shows apparent alternative splicing, which contains two regions of unique nucleotide sequences 1–196 bp and 964–1103 bp, respectively [GenBank: AY943906]. This results in a unique start codon at 378–380 bp and a unique stop codon (TAA) at 1044–1046 bp [GenBank: AY943906]. The open reading frame indicates that putative BCOX1 protein consists of 222 amino acid residues with a molecular weight of 24920 Da and a PI of 5.86 (Fig. 6). Aligning with the deduced amino acid sequences of other KIAA0100-related EST clones, BCOX1 appears to possess a unique 32 amino acid carboxyl-terminus (the italicized letter in Fig. 6). The amino acid sequence analysis of the encoded protein using the PSORT II and TargetP Programs predict this protein is likely a cytoplasmic protein. In addition, BCOX1 contains potential sites for protein kinase C phosphorylation (70–72: SsR; 151–153: SqK; 159–161: TlK), amidation (26–

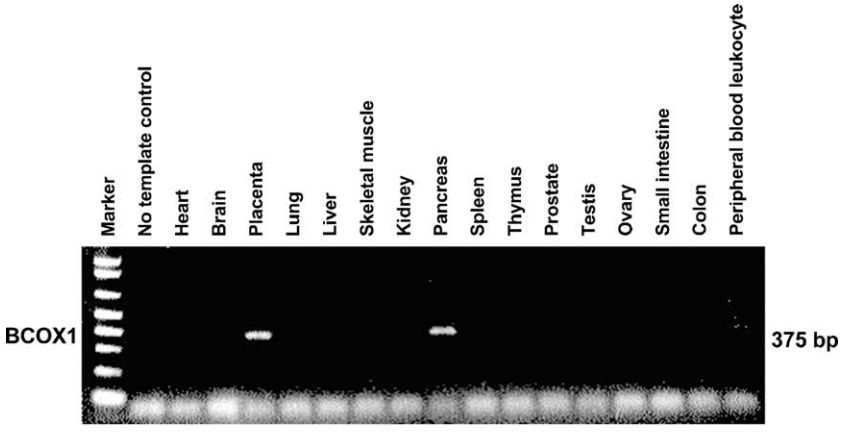

Fig. 5. Restricted expression of BCOX1 in normal tissues. PCR was performed on the panels of cDNA isolated from 16 different human normal tissues (MTC Panel 1 and Panel 2). One specific band with expected size of 375 bp (BCOX1) was detected in placenta and pancreas lanes.

29: dGKR), *N*-myristoylation (91–96: GLfqSQ; 196–201: GSwvGV), and *N*-glycosylation (142–145: NTSV) according to the Prosite database.

4. Discussion

Breast tumorigenesis is a multi-step process starting with benign then atypical hyperproliferation, progressing into in situ carcinomas, then invasive carcinomas, and culminating in metastatic disease [13]. This tumor progression is driven by somatic genetic changes and is reflected in phenotypic changes such as altered gene expression profiles. Therefore, comprehensive and systematic analysis of gene expression profiles may identify key molecular alterations that are important for the acquisition and maintenance of the cancerous phenotype. On the other hand, identification of key molecular alterations may provide useful biomarkers/targets for cancer diagnosis, prognosis, as well as prevention and treatment [14].

In this study, we describe the identification and characterization of a novel tumor-associated gene, BCOX1, in breast carcinomas. Furthermore, RNA in situ hybridization demonstrated that the BCOX1 mRNA expression was absent in normal ductal epithelial cells, moderately elevated in ductal in situ carcinoma cells and peaked in invasive and metastatic breast cancer cells. The BCOX1 signal is mainly located in the cytoplasm of carcinoma epithelial cells, but not in those of normal epithelial cells, stroma cells and lymphocytes. These results suggested: (1) the over-expression of BCOX1 in human breast tissues is cancer cell specific; (2) the over-expression of BCOX1 appears to be correlated with disease progression of breast carcinomas; (3) BCOX1 may be involved in breast tumorigenesis, and could be a promising breast cancer antigen.

BCOX1 cDNA sequence is highly homologous to KIAA0100, a hypothetical gene, located on chromosome 17q11.2. Compared with the KIAA0100-related sequences in humans and other species, BCOX1 shows apparent alternative splicing. The predicted BCOX1 open reading frame of 666 bp encodes a distinct protein of 222 amino acid residues with a molecular weight of 24920 Da and a PI of 5.86. Computational analyses predict that the putative BCOX1 protein is a cytoplasmic protein. Searching the conserved domain database with Reverse Position Specific BLAST (rpsblast) of the NCBI, no any conserved domain was found for both KIAA0100 and BCOX1 protein sequences.

To date, the tissue expression profile and the functional relevance of KIAA0100-related EST variants are yet to be determined. Nagase T., et al. examined tissue expression profile of a variant of KIAA0100 [GenBank: D43947] using human multiple tissue Northern blots (human MTN blots) [12]. This variant was found relative low abundant and no tissue-specific expression. In this study, we identified that the expression profile of BCOX1, but not other KIAA0100 variants, is correlated with the expression profile of SAGE tag of GGTCCCCTAC in breast carcinomas.

In addition, KIAA0100 hypothetical gene was also related to MLAA-22/U937-associated antigen [GenBank: AY288965] due to partial sequence homology. MLAA-22/U937-associated antigen appears to be an immunogenic antigen identified from serum of acute monocytic leukemia [15]. The complete coding sequence (CDS) of MLAA-22/U937 associated antigen is 1896 bp, coding for a protein with 632 amino acids. While both BCOX1 and MLAA-22/U937 reside in the genomic locus of KIAA0100, the CDS of BCOX1 is more than 15,000 bp apart from that of MLAA-22/U937-associated antigen in genomic sequence. Therefore, it is not surprising that BCOX1 shares no sequence homology with MLAA-22/U937-associated antigen. Nevertheless, the co-existence of BCOX1 and MLAA-22/U937-associated antigen in the genomic locus of hypothetical gene KIAA0100 seems to indicate that hypothetical KIAA0100 gene may actually contain more than one functional gene. Due to the distinct tissue expression profile and the unique encoded protein product of BCOX1, we propose a separation of BCOX1 [GenBank: AY943906] from the current hypothetical KIAA0100 gene.

The data obtained in our study showed that BCOX1, a novel gene, was selectively expressed in breast, pancreas and placenta, with overexpression in breast carcinomas. The expression level of BCOX1 appears to be correlated with disease progression of breast carcinomas. This study warrants further investigations to verify BCOX1 as a promising breast cancer antigen. Further understanding the functional and molecular properties of BCOX1 may provide insights to breast tumorigenesis, and BCOX1 may serve as a molecular marker/target for diagnostic and therapeutic applications for breast cancer.

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Expression and clinicopathological significance of oestrogen-responsive ezrin–radixin–moesin-binding phosphoprotein 50 in breast cancer

J Song, J Bai, W Yang, E W Gabrielson, D W Chan & Z Zhang
Department of Pathology, Johns Hopkins Medical Institutions, Baltimore, MD, USA

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Expression and clinicopathological significance of oestrogen-responsive ezrin–radixin–moesin-binding phosphoprotein 50 in breast cancer

Aims: Ezrin–radixin–moesin-binding phosphoprotein 50 (EBP50) is a post synaptic density-95/Disk-large/ZO-1 homologous domain-containing protein that is involved in the linkage of integral membrane proteins to the cytoskeleton and plays an important role in cell signalling. To gain insights into its biological relevance, this study examined expression of EBP50 in two cohorts of breast carcinoma.

Methods and results: Forty-nine breast carcinoma tissue specimens were first examined by both immunohistochemistry and RNA *in situ* hybridization. EBP50 expression was correlated with various clinicopathological variables. The relative abundance of EBP50 mRNA in breast carcinomas and their corresponding normal tissue was compared using reverse transcriptase-polymerase chain reaction (RT-PCR). EBP50 immunoreactivity was then further independently validated in 120 breast carcinomas on tissue microarrays. EBP50 immunoreactivity was observed in morphologically normal and cancerous epithelial cells contrasting with the adjacent immunonegative stromal cells. An elevated cytoplasmic accumulation of EBP50 protein was readily detected in 73.5–80% of breast carcinomas. EBP50 immunoreactivity was significantly associated with tumour stage, lymph node and oestrogen receptor status. These immunohistochemical observations were further validated using RNA *in situ* hybridization and RT-PCR. EBP50 immunoreactivity was significantly correlated with the mRNA expression level.

Conclusion: Oestrogen-responsive EBP50 may play an important role in tumour progression and might be a potential marker of invasiveness for breast cancer.

Keywords: breast cancer, EBP50, immunohistochemistry, oestrogen, RNA *in situ* hybridization

Abbreviations: DAB, diaminobenzidine; DCIS, ductal carcinoma *in situ*; EBP50, ERM-binding phosphoprotein 50; EGFR, epidermal growth factor receptor; ER, oestrogen receptor; ERM, ezrin–radixin–moesin; H&E, haematoxylin and eosin; IBC, invasive breast carcinoma; IHC, immunohistochemistry; ISH, RNA *in situ* hybridization; JHMI, Johns Hopkins Medical Institutions; PDGFR, platelet-derived growth factor receptor; PDZ, post synaptic density-95/Disk-large/ZO-1 homologous; PR, progesterone receptor; RT-PCR, reverse transcriptase-polymerase chain reaction; SPF, S-phase fraction; SSC, sodium saline citrate; TBS–T, Tris-buffered saline–Tween; TMA, tissue microarray; WTS, whole-tissue section

Introduction

Breast cancer is the second leading cause of cancer-related death in women in the USA.[1] The mechanism of breast cancer progression and metastasis is not fully understood. Due to its great heterogeneity, different targeted therapies adapted to each category of breast cancer are therefore required. Among these, whether

Address for correspondence: Zhen Zhang and Jin Song, Department of Pathology, Johns Hopkins Medical Institutions, 419 N. Caroline Str., Baltimore, MD 21231, USA. e-mail: zzhang7@jhmi.edu

or not breast cancers are oestrogen dependent is a critical factor that determines patient prognosis and availability of anti-oestrogenic endocrine therapy.[2,3] Oestrogen receptor (ER)-positive breast cancer generally has a better prognosis and initially responds to anti-oestrogen therapy. However, ER+ breast cancer frequently acquires resistance to endocrine therapy, although ER continues to be expressed.[2,4,5] The molecular mechanisms by which breast cancer becomes hormone refractory are still largely unclear. Identification and functional studies of ER-targeted molecules may provide a clue to understanding the mechanism which alters tumour phenotypes. In addition, although clinicopathological parameters such as tumour stage, grade, lymph node status, ER, progesterone receptor (PR) and HER2/neu expression are helpful, additional breast cancer markers that provide prognostic insights are still needed.

To generate a gene expression-based classification map, one specific tag of GCAGTGGCCT for breast cancer has been identified from the SAGE database.[6] The reliable UniGene cluster matched to this tag is Hs.396783 corresponding to the gene *SLC9A3R1* encoding the solute carrier family 9 (sodium/hydrogen exchanger), member 3 regulator 1. This protein was initially independently identified as ezrin–radixin–moesin (ERM)-binding phosphoprotein 50 (EBP50) or Na^+/H^+ exchanger regulatory factor,[7,8] and will subsequently be referred to in this study as EBP50. EBP50 is a 358-residue adapter molecule (50 kDa) that has two tandem post synaptic density-95/Disk-large/ZO-1 homologous (PDZ) domains followed by a carboxy-terminal ERM-binding region. Through its PDZ domains, EBP50 can bind to many target proteins such as β_2-adrenergic receptor, platelet-derived growth factor receptor (PDGFR) and the cystic fibrosis transmembrane conductance regulator and promote the assembly of membrane-bound macromolecular complexes involved in signal transduction.[9–12] Through its ERM-binding motif, EBP50 can bind to the FERM domain of ERM proteins, which bind to F-actin, involved in cytoskeletal reorganization.[7,13,14] Through these interactions, EBP50 is implicated in the localization of interactive groups of proteins into subcellular domains and in the regulation of activities of those interacting proteins. EBP50 is widely distributed in tissues and is particularly enriched in those containing polarized epithelia.[7,15,16] Recently, EBP50 was further shown to organize ERM proteins at the apical membrane of polarized epithelia.[17] The disruption of epithelial polarity has been thought of as an early step in the development of epithelial neoplasia.[18] More interestingly, EBP50 expression is up-regulated in response to oestrogens and suppressed by anti-oestrogens in ER+ breast cancer cell lines.[19] The correlation between ER status and EBP50 expression has also been observed in breast cancer specimens.[15] These findings suggest a role for EBP50 in the oestrogen signal transduction cascade in oestrogen-responsive tissues.

However, information on the expression of EBP50 in human breast carcinoma tissues is still very limited and the biological significance of EBP50 remains unclear. To gain insight into the biological relevance of EBP50, we first examined expression of EBP50 in 49 cases of breast carcinoma on whole tissue sections (WTS) using immunohistochemistry and RNA *in situ* hybridization (ISH). We then correlated these findings with various clinicopathological variables. The observed clinicopathological associations of EBP50 immunoreactivity were further independently validated in 120 cases of breast carcinoma on tissue microarrays (TMAs).

Materials and methods

HUMAN SUBJECTS AND SPECIMENS

In accordance with the human subject research guidelines of the Institutional Review Board, formalin-fixed paraffin-embedded tissue blocks were retrieved from the Department of Pathology at Johns Hopkins Medical Institutions (JHMI). The first cohort of breast tissue specimens included 12 ductal carcinomas *in situ* (DCIS) and 37 invasive breast carcinomas (IBC), which were obtained from patients who underwent reduction mammoplasty or mastectomy during the period 1997–2003. For each specimen selected, one section was subjected to haematoxylin and eosin (H&E) staining for routine examination and others were processed for immunohistochemistry (IHC) and ISH analysis. The second cohort of breast tissue specimens included 120 IBCs on the TMAs, which were obtained from patients who underwent reduction mammoplasty or mastectomy during the period 1991–1993. Available tumour clinicopathological characteristics include age, tumour size, stage, histological type, Elston grade and lymph node status. Some cases were assessed for biological parameters, including steroid hormone receptors (ER/PR), HER2/neu status, Ki67 index, DNA index, ploidy and S-phase fraction (SPF). The breast carcinomas were classified according to the 1999 World Health Organization criteria.

TMA CONSTRUCTION

TMAs were constructed as described previously.[20,21] Briefly, tissue cores (1.5 mm in diameter) were taken

twice from spatially separate areas in a single donor block from each case using a tissue microarrayer (Beecher Instruments, Silver Spring, MD, USA). Cores were precisely arrayed into a recipient paraffin block at defined coordinates to form an array of 11 × 9 cores format. The H&E-stained sections from donor and recipient paraffin blocks were used to identify or confirm the area of tumour from which cores were retrieved or the presence of tumour on TMAs, respectively.

IMMUNOHISTOCHEMISTRY

The EnVision+System-horseradish peroxidase [diaminobenzidine (DAB)] (DakoCytomation, Carpinteria, CA, USA) was used in this study. Four-micrometre histological sections (WTS and TMA sections) were deparaffinized and rehydrated in graded ethanol. Endogenous peroxidase activity was blocked by 0.03% hydrogen peroxide for 10 min. Antigen retrieval was performed in 10 mM sodium citrate buffer (pH 6.0) for 15 min. Sections were then incubated with rabbit polyclonal antihuman EBP50 antibody (PA1-090; Affinity BioReagents, Golden, CO, USA) (1 : 800 dilution) at room temperature for 40 min. After incubation, specimens were washed with 1× Tris-buffered saline–Tween (TBS–T) buffer (DakoCytomation) and incubated with peroxidase-labelled polymer at room temperature for 30 min. The samples were then washed with 1× TBS–T buffer and incubated with freshly prepared DAB + substrate–chromogen buffer at room temperature for 8 min. After gently rinsing with dH_2O, slides were counterstained with haematoxylin (DakoCytomation) and mounted with permanent mounting media (DakoCytomation). For negative controls, the primary antibody was omitted and replaced by preimmune rabbit serum (I8140; Sigma, St Louis, MO, USA).

REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION

The template was cDNA from five pairs of human breast carcinomas and corresponding normal tissue of individual patients (Human Breast Matched cDNA Pair Panel; Clontech, Palo Alto, CA, USA). Taq DNA polymerase was purchased from Promega Corporation (Madison, WI, USA). Polymerase chain reaction (PCR) amplifications were carried out in a Mastercycler (Eppendorf, Westbury, NY, USA) as follows: initial denaturation at 94°C for 5 min; 35 cycles of 40 s at 94°C, 1 min at 56.8°C and 1.5 min at 72°C; followed by a final 5-min extension at 72°C. Amplified PCR products were electrophoresed on a 1% agarose gel and visualized by ethidium bromide staining.

The primer design for PCR was as follows: EBP50 PCR product of 434 bp, upper primer 5′-ATC GCA TTG TGG AGG TGA-3′, lower primer 5′-TTT GCT GCT GCG TTT CT-3′; β-actin PCR product of 252 bp, upper primer 5′-AGG CAT CCT CAC CCT GAA GT-3′, lower primer 5′-CCA GAG GCG TAC AGG GAT A-3′. All primers were purchased from Qiagen (Valencia, CA, USA).

PREPARATION OF SENSE AND ANTISENSE EBP50 PROBES

The EBP50 PCR product of 434 bp from the above reverse transcriptase (RT)-PCR for the Breast Matched cDNA Pair Panel was purified using the QIAquick PCR Purification Kit (Qiagen). This purified cDNA fragment was subcloned into the transcription vector pCR®II-TOPO® (Invitrogen, Carlsbad, CA, USA) and transformation of TOP10 cells was performed according to the manufacturer's protocol. Several clones were analysed for the presence of an EBP50 insert by digestion with EcoRI (Invitrogen) on the purified plasmid DNA (QIAprep Spin Miniprep kit; Qiagen). Automated sequencing of the representative inserts was performed at the DNA Core Facility at JHMI. To generate the antisense (complementary to EBP50 mRNA) and sense RNA probes, the purified plasmid DNA was first linearized with XhoI or BamHI (New England BioLabs Inc., Beverly, MA, USA). Antisense and sense RNA probes labelled with digoxigenin-UTP (Roche Diagnostics Corp., Indianapolis, IN, USA) were generated by *in vitro* transcription of purified linearized plasmids (QIAquick PCR Purification Kit; Qiagen) with SP6 or T7 RNA polymerase (Roche Diagnostics) according to the manufacturer's protocol. All other chemicals of molecular biology grade were obtained from Sigma-Aldrich.

RNA *IN SITU* HYBRIDIZATION

ISH was carried out using a non-radioactive method as reported previously, with some modifications.[22] To control signal specificity, two consecutive sections were used for hybridization with the antisense and sense probes, respectively. Briefly, 4-μm histological sections were deparaffinized and rehydrated in graded ethanol. The sections were subjected to protease K digestion (10 ng/μl) at 37°C for 30 min. The slides were washed briefly with TBS buffer (pH 7.5) and equilibrated with 100 μl prehybridization solution without probe at 45°C for 1 h. The prehybridization solution contained 50% formamide, 4× sodium saline citrate (SSC), 2×

Denhardt's solution, 5× dextran sulphate, 0.1% sodium dodecyl sulphate and 400 μg/ml denatured salmon sperm DNA. The 50-μl hybridization solution containing 1 ng/μl probe (sense or antisense) was then applied to each section. The slides were covered with a coverslip and placed on a heating block at 65°C for 5 min to eliminate hairpin loops or other secondary structures of the mRNA sequences. Hybridization was carried out at 55°C overnight in a humidified chamber. The slides were washed with 2× SSC twice for 30 min followed by washing once in 2× SSC at 55°C for 20 min.

Detection of *in situ* hybridization probes was performed using antidigoxigenin–alkaline phosphatase system (Roche Diagnostics).[22] Briefly, after equilibration in TBS–T buffer for 5 min, the sections were incubated with 1× blocking buffer (Roche) at room temperature for 30 min. The slides were then incubated with an antibody solution containing 1 : 500 diluted alkaline phosphatase-conjugated Fab fragment from a sheep antidigoxigenin antibody (Roche) at room temperature for 1 h 30 min. The sections were washed three times with TBS–T buffer for 10 min and equilibrated in TBS buffer (pH 9.5) at room temperature for 5 min to activate alkaline phosphatase. Colorimetric detection of the digoxigenin-labelled probe was performed by applying 100 μl of substrate solution containing Nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate per slide and incubation at room temperature for 4 h in a humidified chamber. Colour development was terminated by two washes of 3 min each in nuclease-free water. The sections were dehydrated and mounted with the supermount medium (Fisher Scientific, Fairlawn, NJ, USA). A positive enzyme reaction in this assay stained dark purple.

EVALUATION OF IHC AND ISH

EBP50 IHC and ISH results were scored semiquantitatively from 0 to 3+ as follows: negative (0), weak (1+), moderate (2+) and strong (3+) expression. Briefly, both the percentage of stained cells (0, ≤ 10%; 1, 11–25%; 2, 26–50%; 3, 51–75%; 4, 76–90%; 5, ≥ 91%) and the intensity of the staining (0, none; 1, weak; 2, moderate; 3, strong) were assessed in every component on every slide as described previously.[23,24] A final score was obtained by combining the percentage of stained cells with the intensity of the staining as follows: samples with an intensity of 0 or 1 in ≤ 10% of cells were designated negative; samples with an intensity of 1 in > 10% of cells or with an intensity of 2–3 and the added scores of 2–3 were designated weak; samples with an intensity of 2–3 and the added scores of 4–6 or 7–8 were designated moderate or strong, accordingly. Each core on TMAs was scored individually and then the final score for each case was determined by combining the results of duplicate scores for that case.

Thirty-seven cases in the first cohort (WTS) were assessed semiquantitatively for ER and PR status using IHC. Samples with any intensity level in ≥ 10% of cells or moderate and strong intensity in < 10% of cells were designated positive; samples with weak intensity in < 10% of cells or without staining were designated negative. One hundred and thirteen cases in the second cohort (TMAs) were assessed quantitatively for ER and PR concentrations using an enzyme immunoassay. The cut-off for positivity was > 10 fmol/mg for both ER and PR. Samples with a Ki67 index of < 10% were designated low, 10–30% as median and > 30% as high. Staining of HER2/neu (Dako HercepTest kit) was scored semiquantitatively from 0 to 3+ as described previously:[25] 0 or 1+ were considered negative, 2+ weakly positive and 3+ strongly positive. To qualify for 2+ and 3+ scoring (i.e. positive), complete membranous staining of > 10% of tumour cells was necessary.

STATISTICAL ANALYSIS

The PCR band densities were measured using Quantity One-version 4.5 (Bio-Rad, Hercules, CA, USA) and Student's *t*-test was used to analyse the differential expression levels of EBP50 between the paired breast carcinomas and their corresponding normal controls. One-way ANOVA or cross-table χ^2 tests were performed to compare the EBP50 differential expression levels (IHC and ISH) in breast carcinomas. Differences with $P < 0.05$ were considered to be statistically significant. All statistical analysis was performed using Statistica 6.1 (Statsoft, Tulsa, OK, USA).

Results

IMMUNOHISTOCHEMISTRY FOR EBP50 EXPRESSION IN BREAST CARCINOMA TISSUES

Out of 49 cases of breast carcinoma tissues in the first cohort (WTS), 36 (73.5%) were EBP50 immunopositive, whereas 13 were EBP50 immunonegative. More specifically, 13 (26.5%) were negative, 15 (30.6%) were weakly and 21 (42.9%) were moderately/strongly immunopositive. Among the EBP50-immunopositive cases, immunopositivity was observed in morphologically normal and cancerous epithelial cells, contrasting with the adjacent immunonegative stromal cells (Figure 1). In morphologically normal epithelial cells, EBP50 positivity was present mostly as apical

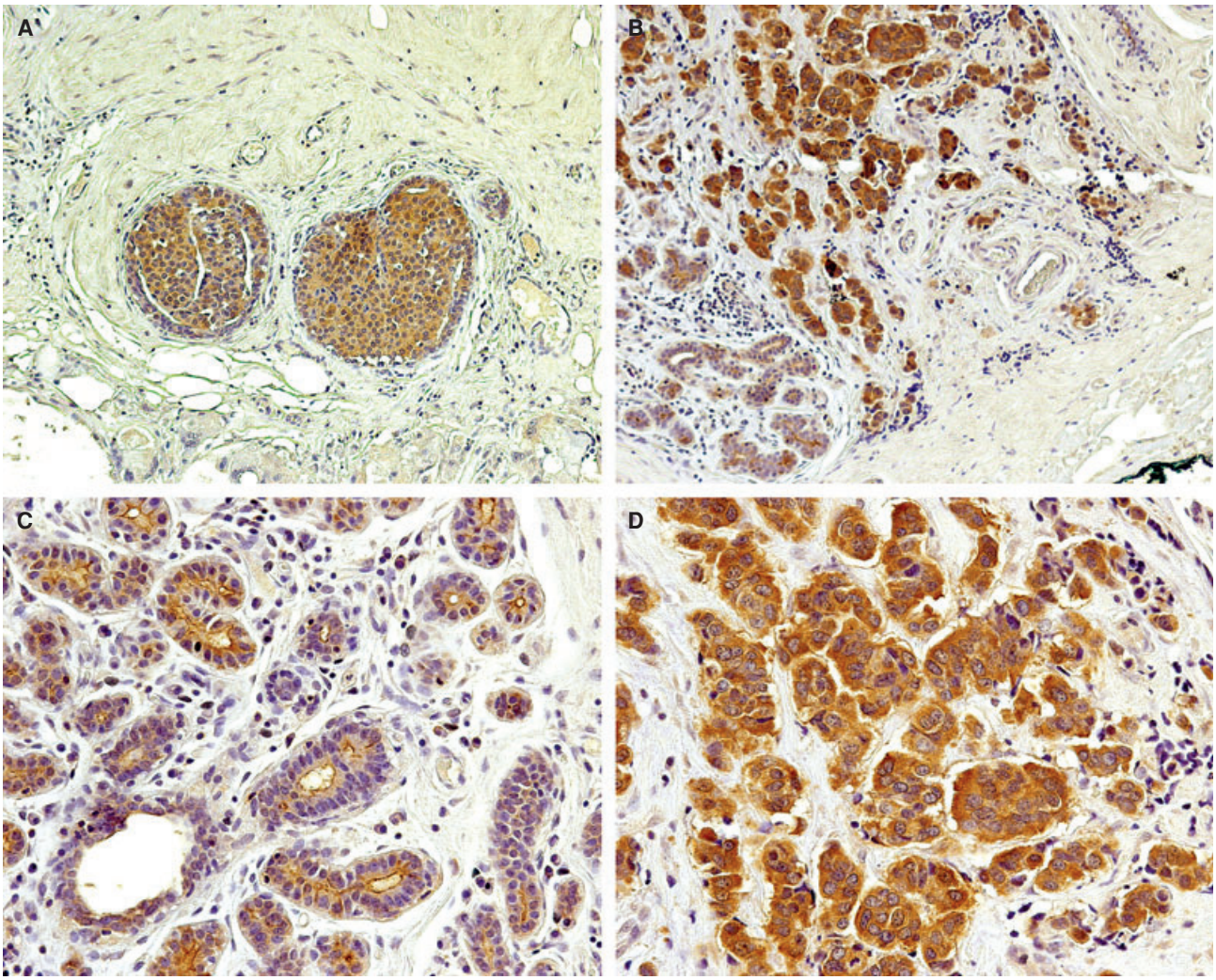

Figure 1. Immunoreactivity and localization of ERM-binding phosphoprotein 50 (EBP50) in breast carcinoma. EBP50 immunoreactivity in invasive breast carcinoma (**B**) was much greater than that in ductal carcinoma *in situ* (**A**). EBP50 positivity was present mostly as apical membranous immunoreactivity (**C**) or cytoplasmic accumulation (**D**) in morphologically normal (**C**) or cancerous (**D**) epithelial cells, respectively (**C**,**D**, same case from **B**).

membranous staining (Figure 1B,C). In cancerous epithelial cells, an increase in the amount of cytoplasmic EBP50 protein was readily detected compared with levels in the surrounding non-cancerous epithelial cells and EBP50 positivity was present mostly as cytoplasmic accumulation (Figure 1B,D). No significant EBP50 immunoreactivity was detected in negative controls that were analysed with the primary antibody omitted and preimmune serum (data not shown).

CLINICOPATHOLOGICAL ASSOCIATIONS OF EBP50 IMMUNOREACTIVITY IN BREAST CARCINOMAS

The associations between EBP50 immunoreactivity and clinicopathological variables in 49 breast carcinomas of the first cohort are summarized in Table 1. EBP50 immunoreactivity was statistically significantly associated with stage ($P = 0.0102$), lymph node status ($P = 0.0006$) and ER status ($P = 0.0003$) (also see Figure 1A,B and 2A–F). Notably, there was a significant difference in EBP50 expression level between IBC and DCIS ($P = 0.0154$) (see also Figure 1A,B). In contrast to the 27 of 29 (93.1%) ER+ breast carcinomas were EBP50 immunopositive (Figure 2A,B,D,E), 5 of 8 ER– breast carcinomas were EBP50 immunonegative (Figure 2C,F). Of the 27 with both EBP50+ and ER+ tumours, 20 (74.1%) showed moderate or strong EBP50 staining (Figure 1D). A negative correlation was observed between EBP50 immunoreactivity and patient's age ($P = 0.0126$, weak versus moderate/

Table 1. Clinicopathological associations of ERM-binding phosphoprotein 50 immunoreactivity in breast carcinomas on whole tissue sections

Variables	*n*	Negative	Weak	Moderate/strong	*P*
Age* (years)	49	55.1 ± 7.6	60.1 ± 7.7	48.6 ± 5.6	0.0126 (N versus M/S)
Tumour size* (mm)	49	20.6 ± 7.1	23.8 ± 14.8	29.2 ± 6.9	NS
Stage					
0	12	6 (50%)	5 (41.7%)	1 (8.3%)	
I	13	6 (46.1%)	2 (15.4%)	5 (38.5%)	
II	16	1 (6.2%)	6 (37.5%)	9 (56.3%)	
III	8	0 (0%)	2 (25%)	6 (75%)	0.0102
DCIS	12	6 (50%)	5 (41.7%)	1 (8.3%)	
IBC	37	7 (18.9%)	10 (27%)	20 (54.1%)	0.0154
Histotype					
Ductal	44	13 (29.5%)	11 (25%)	20 (45.5%)	
Lobular	5	0 (0%)	4 (80%)	1 (20%)	NS
Elston grade					
I	5	1 (20%)	2 (40%)	2 (40%)	
II	25	4 (16%)	6 (24%)	15 (60%)	
III	19	8 (42.1%)	7 (36.8%)	4 (21.1%)	NS
Lymph node status					
N–	28	13 (46.4%)	8 (28.6%)	7 (25%)	
N+	21	0 (0%)	7 (33.3%)	14 (66.7%)	0.0006
Steroid receptors					
ER–	8	5 (62.5%)	3 (37.5%)	0 (0%)	
ER+	29	2 (6.9%)	7 (24.1%)	20 (69%)	0.0003
PR–	18	5 (27.8%)	6 (33.3%)	7 (38.9%)	
PR+	19	2 (10.5%)	4 (21.1%)	13 (68.4%)	NS
Ki67 index					
Low	7	2 (28.6%)	1 (14.3%)	4 (57.1%)	
Median	19	3 (15.8%)	5 (26.3%)	11 (57.9%)	
High	9	2 (22.2%)	3 (33.3%)	4 (44.4%)	NS
HER2/neu status					
Negative	25	5 (20%)	6 (24%)	14 (56%)	
Weak	3	0 (0%)	1 (33.3%)	2 (66.7%)	
Strong	4	1 (25%)	0 (0%)	3 (75%)	NS

*Data are presented as mean ± 95% confidence interval. All other values represent the number of cases and percentage. The *P*-value was calculated using one-way ANOVA or a cross-table χ^2 test. Differences with $P < 0.05$ were considered to be significant.
NS, Not significant; DCIS, ductal carcinoma *in situ*; IBC, invasive breast carcinoma; ER, oestrogen receptor; PR, progesterone receptor.

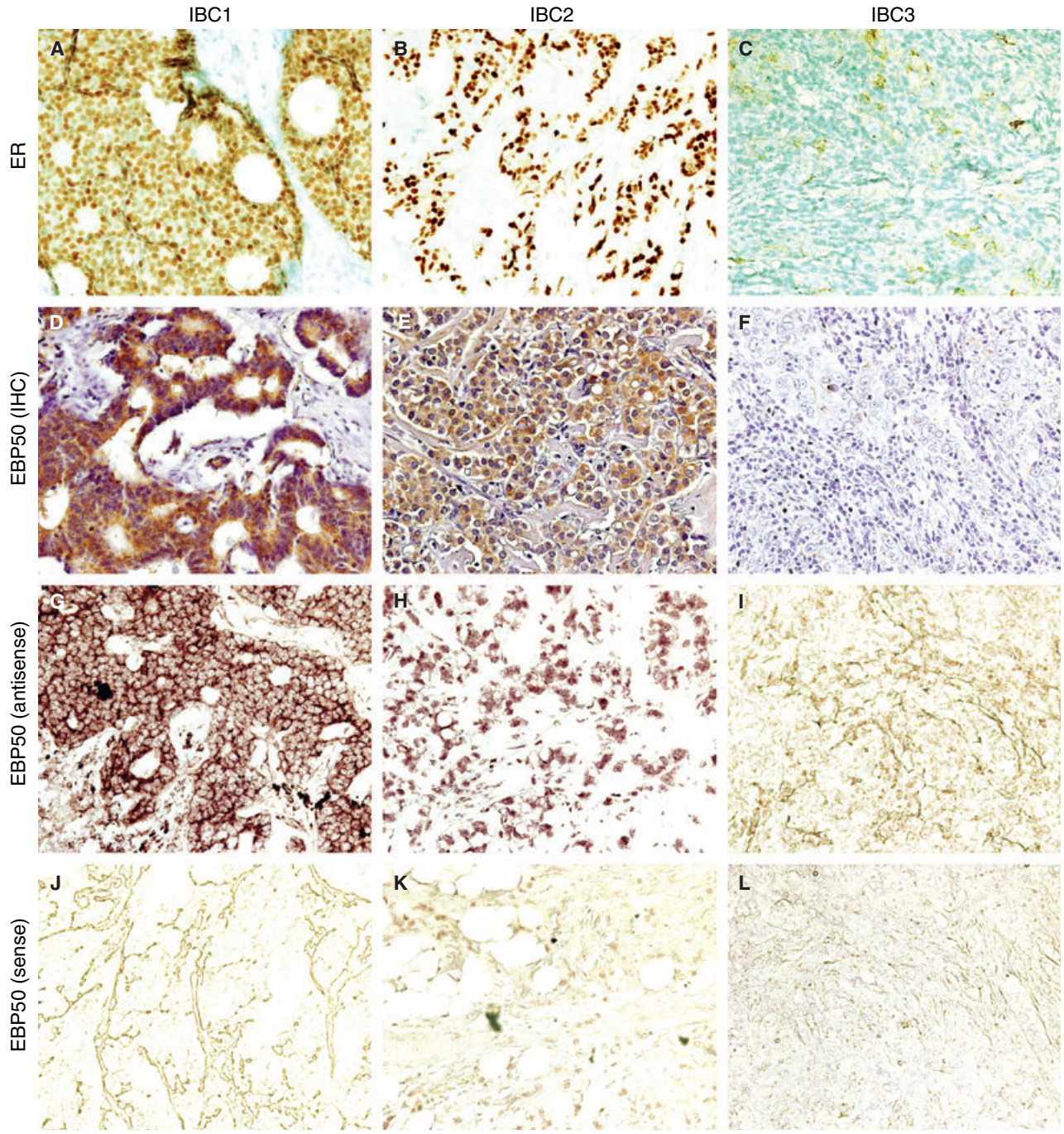

Figure 2. Correlation of ERM-binding phosphoprotein 50 (EBP50) immunoreactivity and mRNA expression in breast carcinoma. Examples of EBP50 protein and mRNA expression in oestrogen receptor (ER)-positive (IBC1 and IBC2) and ER– (IBC3) breast carcinomas. IBC1, with tumour involved in lymph nodes (N+); IBC2 and IBC3, without tumour involved in lymph nodes (N–). Staining in **A**, **B** and **C** represents immunohistochemical detection of ER protein. Strong (**D**) and weak (**E**) EBP50 immunoreactivity is found in IBC1 and IBC2, respectively, whereas negative staining (**F**) is indicated in IBC3. Similar patterns of immunoreactivity with EBP50 antisense probe are seen in **G** (strong), **H** (weak) and **I** (negative), suggesting that the mRNA expression of EBP50 correlates with its immunoreactivity in these tissue specimens. An EBP50 sense probe was used as a negative control for every specimen by hybridization on a consecutive section (no signals in **J**, **K** and **L**).

strong) in this cohort. However, there was no significant relationship observed in this cohort between EBP50 immunoreactivity and other clinicopathological variables, including tumour size, histological type, Elston grade, PR status, Ki67 index and HER2/neu status.

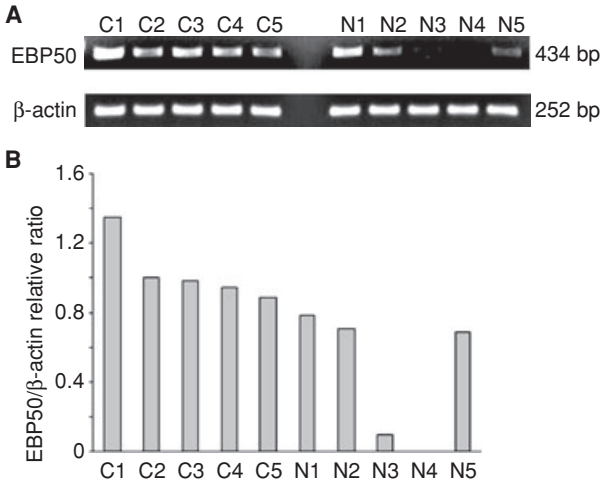

Figure 3. ERM-binding phosphoprotein 50 (EBP50) mRNA expression in breast carcinoma and corresponding normal tissue. **A,** Reverse transcriptase-polymerase chain reaction was performed on a panel of cDNA isolated from five matched tissue pairs of human breast carcinoma and corresponding normal tissue. The expression levels of EBP50 mRNA (the specific band at 434 bp) in breast carcinomas (lanes C1–C5) are much higher than those of corresponding normal breast tissues (lanes N1–N5). The expression level of β-actin (the band at 252 bp) was taken as a loading control for this comparison. **B,** The relative ratios of EBP50/β-actin expression levels (band densities) are shown in the histogram. Student's *t*-test shows a significant difference in expression levels of EBP50 mRNA between the paired breast carcinomas and their corresponding normal partners ($P = 0.01$).

EXPRESSION OF EBP50 mRNA IN BREAST CARCINOMAS AND CORRESPONDING NORMAL TISSUES

To determine the relative abundance of EBP50 mRNA in breast carcinomas and their corresponding normal tissues regardless of ER status, we performed RT-PCR on a panel of cDNA isolated from five matched tissue pairs of human breast carcinoma and corresponding normal tissue. As shown in Figure 3A,B, the expression levels of EBP50 mRNA (the specific band at 434 bp) in breast carcinomas (lanes C1 to C5) were much higher than those in corresponding normal breast tissue (lanes N1 to N5), respectively. The expression level of β-actin (the band at 252 bp) was taken as a loading control for this comparison. Statistical analysis based on the relative ratios of EBP50/β-actin (band densities) showed that there was a significant difference in expression levels of EBP50 mRNA between the paired breast carcinomas and their corresponding normal partners ($P = 0.01$).

RNA *IN SITU* HYBRIDIZATION ANALYSIS

Furthermore, ISH analyses were performed on the same cohort (WTS) of 49 breast carcinoma tissues. EBP50 mRNA expression was visualized by ISH using a digoxigenin-labelled EBP50 antisense probe. EBP50 sense probe was used as a negative control for every specimen by hybridization on a consecutive section. The experimental results showed apparently cytoplasmic reactivity in breast carcinoma epithelial cells, but not in stromal cells or lymphocytes (Figure 2G,H). No specific signal was observed in controls hybridized with the EBP50 sense probe (Figure 2J,K). As summarized in Table 2, EBP50 mRNA expression was also significantly associated with stage ($P = 0.0146$), lymph node status ($P = 0.0008$) and ER status ($P = 0.0054$) (also see Figure 2G–L). In contrast, there was no significant relationship between EBP50 mRNA expression and the remaining clinicopathological variables, including patient's age, tumour size, histological type, Elston grade, PR status, Ki67 index and HER2/neu status (data not shown). As shown in Table 3 and Figure 2D–I, there was a significant correlation between EBP50 immunoreactivity (IHC) and EBP50 mRNA expression (ISH) levels in this cohort of breast carcinomas ($P = 0.0021$). Overall, the ISH results were in agreement with the immunohistochemistry data and confirmed that expression of oestrogen-responsive EBP50 was strongly associated with several clinicopathological features of breast carcinoma.

INDEPENDENT VALIDATION OF THE IMMUNOHISTOCHEMICAL STUDY

As an independent validation of the above immunohistochemical observations, we further examined EBP50 immunoreactivity in 120 cases of breast carcinoma, the second cohort, on TMAs. Of 120 cases, 96 (80%) were EBP50 immunopositive, whereas 24 were EBP50 immunonegative. More specifically, 24 (20%) were negative, 33 (27.5%) were weakly and 63 (52.5%) were moderately/strongly immunopositive. The expression pattern and cellular localization of EBP50 immunoreactivity on TMAs were demonstrated to be the same as those on the WTS (Figure 4A–C). As shown in Table 4, EBP50 immunoreactivity was statistically significantly associated with stage ($P = 0.0003$), lymph node status ($P = 0.0043$) and ER status ($P = 0.0000$) or ER concentration ($P = 0.018$, negative versus weak; $P = 0.0000$, negative versus moderate/strong; $P = 0.0423$, weak versus moderate/strong). In contrast to 62 of 64 (96.9%) ER+ breast carcinomas were EBP50 immunopositive, 21 of 49 (43%) ER– breast carcinomas were EBP50 immunonegative (Figure 4A–C). Of the 62 with both EBP50+ and ER+ tumours, 45 (72.3%) showed moderate or strong EBP50 immunoreactivity. Although

Table 2. ERM-binding phosphoprotein 50 (EBP50) mRNA expression evaluated by *in situ* hybridization on whole tissue sections

Variables	*n*	Negative	Weak	Moderate/ strong	*P*
Stage					
0	12	7 (58.3%)	5 (41.7%)	0 (0%)	
I	13	6 (46.2%)	4 (30.8%)	3 (23%)	
II	16	2 (12.5%)	4 (25%)	10 (62.5%)	
III	8	2 (25%)	1 (12.5%)	5 (62.5%)	0.0146
Lymph node status					
N–	28	13 (46.4%)	11 (39.3%)	4 (14.3%)	
N+	21	4 (19%)	3 (14.3%)	14 (66.7%)	0.0008
Steroid receptors					
ER–	8	5 (62.5%)	3 (37.5%)	0 (0%)	
ER+	29	5 (17.2%)	6 (20.7%)	18 (62.1%)	0.0054

All values represent the number of cases and percentage. The *P*-value was calculated using a cross-table χ^2 test. Differences with $P < 0.05$ were considered significant.
ER, Oestrogen receptor; PR, progesterone receptor.

Table 3. Correlation of ERM-binding phosphoprotein 50 (EBP50) immunoreactivity and mRNA expression in breast carcinomas on whole tissue sections

	EBP50 mRNA level		
	Negative (17)	Weak (14)	Moderate/ strong (18)
EBP50 immunoreactivity			
Negative (13)	8 (61.5%)	4 (30.8%)	1 (7.7%)
Weak (15)	3 (20%)	8 (53.3%)	4 (26.7%)
Moderate/strong (21)	6 (28.6%)	2 (9.5%)	13 (61.9%)

All values represent the number of cases and percentage. *P*-value was calculated using a cross-table χ^2 test. There was a significant correlation between EBP50 immunoreactivity (immunohistochemistry) and EBP50 mRNA expression (*in situ* hybridization) levels in breast carcinomas ($P = 0.0021$).

EBP50 immunoreactivity was also shown to be associated with PR status ($P = 0.0093$) at the positive cut-off of >10 fmol/mg, it was not correlated with PR concentration (Table 4). In contrast to the observation in the first cohort, EBP50 immunoreactivity was not associated with patient's age in the second cohort. Moreover, there was no significant relationship observed between EBP50 immunoreactivity and other clinicopathological variables, including tumour size, histological type, DNA index, ploidy and SPF in this cohort. There were incomplete data in some cases in this cohort on such parameters as tumour size, stage, Elston grade and lymph node status, resulting in a different total number of cases being analysed in Table 4. Due to the low number of cases with Elston grade, this parameter is not included in Table 4.

Discussion

EBP50 is highly expressed in epithelia of many tissues, particularly in cells with numerous microvilli, and is often concentrated at the apical membrane of those cells.[7,15,16] Among various human tissues examined, EBP50 was found to be expressed at a fairly high level in mammary tissue.[19] Several studies have demonstrated that EBP50 is a physiologically relevant ERM-binding phosphoprotein that is involved in the linkage of integral membrane proteins to the cytoskeleton.[7,9–14] It is therefore believed to play an important role in cell signalling associated with changes in cell cytoarchitecture. The polarized architecture of epithelia is a basic property of human epithelial tissues which makes possible unidirectional fluid and solute transport. The disruption of epithelial polarity has been thought of as an early stage in the development of the epithelial neoplasm.[18] In this study, we first examined expression of EBP50 in 49 cases of breast carcinoma on WTS using immunohistochemistry and ISH. EBP50 immunoreactivity was detected in 36 of 49 human breast carcinomas (73.5%) and was significantly correlated with EBP50 mRNA expression level. EBP50 immunoreactivity was detected in both

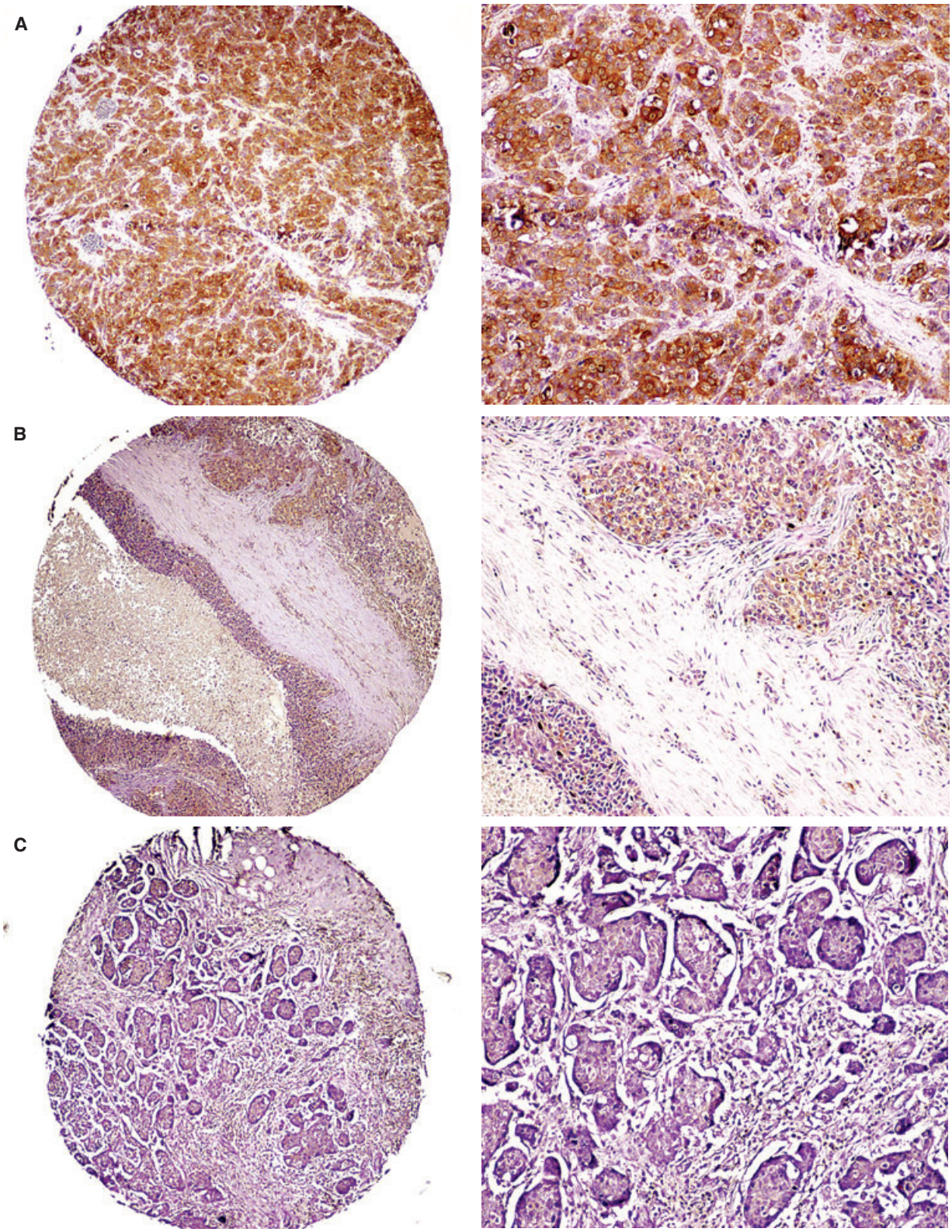

Figure 4. Independent validation of immunohistochemical study on tissue microarrays. Examples of ERM-binding phosphoprotein 50 (EBP50) protein expression in oestrogen receptor (ER)-positive (**A**,**B**) and ER– (**C**) breast carcinoma. Strong and weak EBP50 immunoreactivity is found in cases **A** and **B**, respectively, whereas negative staining is indicated in case **C**.

Table 4. Clinicopathological associations of ERM-binding phosphoprotein 50 immunoreactivity in breast carcinomas on tissue microarrays

Variables	*n*	Negative	Weak	Moderate/strong	*P*
Age* (years)	120	54.1 ± 4.0	52.8 ± 5.0	54.3 ± 3.5	NS
Tumour size* (mm)	94	34.3 ± 12.1	35.4 ± 9.8	33.5 ± 6.3	NS
Stage					
I	9	6 (66.7%)	2 (22.2%)	1 (11.1%)	
II	28	6 (21.4%)	9 (32.1%)	13 (46.5%)	
III	43	4 (9.3%)	6 (14%)	33 (76.7%)	0.0003
Histotype					
Ductal	110	22 (20%)	29 (26.4%)	59 (53.6%)	
Lobular	10	2 (20%)	4 (40%)	4 (40%)	NS
Lymph node status					
N–	22	9 (40.9%)	6 (26.3%)	7 (31.8%)	
N+	58	7 (12%)	11 (19%)	40 (69%)	0.0043
Steroid receptors					
ER–	49	21 (43%)	14 (28.5%)	14 (28.5%)	
ER+	64	2 (3.1%)	17 (26.6%)	45 (70.3%)	0.0000
ER (fmol/mg)		16.8 ± 19.4	74.7 ± 48.7	138.4 ± 52.4	0.018 (N versus W) 0.0000 (N versus M/S) 0.0423 (W versus M/S)
PR–	58	18 (31%)	16 (27.6%)	24 (41.4%)	
PR+	55	5 (9.1%)	15 (27.3%)	35 (63.6%)	0.0093
PR (fmol/mg)		78 ± 99.1	118.6 ± 100.7	161.5 ± 61.8	NS
DNA ploidy					
Diploid	28	3 (10.7%)	8 (28.6%)	17 (60.7%)	
Aneuploid	83	19 (22.9%)	23 (27.7%)	41 (49.4%)	NS
DNA index		1.52 ± 0.15	1.53 ± 0.16	1.49 ± 0.11	NS
S-phase fraction	70	8.8% ± 3.6%	7.9% ± 2.7%	6.2% ± 1.5%	NS

*Data are presented as mean ± 95% confidence interval. All other values represent the number of cases and percentage. The *P*-value was calculated using one-way ANOVA or a cross-table χ^2 test. Differences with $P < 0.05$ were considered significant. NS, Not significant; ER, oestrogen receptor; PR, progesterone receptor.

morphologically normal and cancerous epithelial cells, but not in adjacent stromal cells. In morphologically normal epithelial cells, EBP50 immunoreactivity was observed mainly in apical membranes of cells. In cancerous epithelial cells, in addition to such membranous localization, increased cytoplasmic accumulation of EBP50 protein was readily detected compared with the surrounding non-cancerous epithelial cells. As an independent validation of the immunohistochemical observations, we further examined EBP50 immunoreactivity in 120 cases of breast carcinoma on TMAs. The expression pattern and cellular localization of EBP50 immunoreactivity with a positivity of 80% (96/120) on TMAs were the same as those on

WTS. Moreover, RT-PCR analysis indicated that there was a significant difference in expression level of EBP50 mRNA between the paired breast carcinomas and their corresponding normal partners. EBP50 has been reported to be expressed in 16 of 18 human breast carcinoma tissues (66.7%) using immunohistochemistry and its immunopositivity as being membranous, especially at the luminal aspect of cells, and as diffusely cytoplasmic.[15] The cytoplasmic and nuclear accumulation of EBP50 protein has also been shown in 21 of 38 human hepatocellular carcinomas (55%) compared with surrounding non-cancerous liver tissue.[26] Northern blot and RT-PCR analysis have further revealed an increase of EBP50 mRNA in hepatocellular carcinoma cell lines and surgical specimens of human hepatocellular carcinoma.[26] In contrast to these observations, intragenic mutation of the *EBP50* gene accompanied by loss of heterozygosity was found in ~3% (3/85) of breast cancer cell lines and primary breast tumours,[27] indicating the highly heterogeneous nature of this disease. The biological relevance of EBP50 in breast carcinoma, therefore, required further investigation. The frequency and cellular localization of EBP50 immunoreactivity in our study were in good agreement with previous reports[15,26] and the elevated cytoplasmic accumulation of EBP50 in breast carcinoma suggests that it has an important role in the development and progression of the disease.

It is well known that oestrogens influence cytoarchitecture and influence several cell signalling pathways, such as epidermal growth factor and tyrosine kinase/MAP-kinase pathways.[28–33] They regulate a variety of biological processes in ER+ breast cancer cells, including cell proliferation and invasiveness.[34,35] However, the nature of the links between the oestrogen signalling pathway and other cell signalling pathways is not well understood. EBP50 expression has been reported to be up-regulated by oestrogens and suppressed by anti-oestrogens in ER+ breast cancer cells.[19] The correlation between ER status and EBP50 immunoreactivity has also been observed in breast tumours.[15] In concordance with these results, our experimental data have shown that both EBP50 mRNA and protein expression were strongly associated with ER status in 49 human breast carcinoma tissues. The association between EBP50 immunoreactivity and ER status was further independently validated in 120 cases of breast carcinoma. Therefore, it can be hypothesized that EBP50 plays a role in oestrogen-mediated cell growth in breast carcinoma and is a possible link whereby the different pathways communicate, as it has been shown to participate in several relevant aspects of signal transduction.[9–12,36] For example, EBP50 has been shown to bind the C terminus of epidermal growth factor receptor (EGFR), stabilize EGFR at the cell surface and prevent its agonist-induced receptor down-regulation.[36] EBP50 has been found to associate with PDGFR and potentiate the receptor's signalling activity.[10] These different signalling pathways extensively cross-talk and overlap with each other and may partly explain how ER+ breast cancers adapt to and bypass anti-oestrogen therapy and become hormone refractory. It is also possible that an alternative pathway for oestrogen signalling in ER– breast cancers involves EBP50 expression via ER-independent up-regulation, which could explain the 37.5–56% of ER– breast carcinomas expressing EBP50 in our series.

Breast tumorigenesis is a multistep process starting with benign then atypical hyperproliferation, progressing to *in situ* carcinoma, then invasive carcinoma, and culminating in metastatic disease.[37] For example, DCIS is generally regarded as a precursor to IBC, although not all DCIS lesions progress to invasiveness during a patient's lifetime.[38] However, the progression from *in situ* to invasive carcinoma is poorly understood. It would be relevant to identify more promising tumour markers that could provide important prognostic insights. In this study, EBP50 immunoreactivity was statistically significantly associated with tumour stage, lymph node invasion and ER status in two cohorts of breast carcinoma. Notably, there was also a significant difference in EBP50 protein expression level between IBC and DCIS in the first cohort. The observations of immunohistochemistry were further validated using ISH analysis. EBP50 immunoreactivity was significantly correlated with EBP50 mRNA expression level in the first cohort of breast carcinomas. These results suggest that oestrogen-responsive EBP50 expression is strongly associated with several malignant clinicopathological features of breast carcinoma, and stronger EBP50 expression reflects greater tumour invasiveness.

In summary, this is the first large-scale study of immunohistochemistry and RNA *in situ* hybridization analysis of oestrogen-responsive EBP50 expression in a series of breast carcinomas and correlation of its expression with various clinicopathological variables. Elevated cytoplasmic accumulation of EBP50 protein was readily detected in 73.5–80% of breast carcinoma tissues and was significantly correlated with EBP50 mRNA expression level. EBP50 immunoreactivity was significantly associated with tumour stage, lymph node status and ER status. These results suggest that oestrogen-responsive EBP50 might be a potentially

promising marker of invasiveness for breast cancer. Furthermore, given its obvious abundance in most of the invasive tumours examined and its potential role in oestrogen-mediated tumour growth, EBP50 may represent a novel subcellular therapeutic target in breast cancer.

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